Helix-Folding Templates

A Cyclic Metallopeptide Induces α Helicity in Short Peptide Fragments of Thermolysin**

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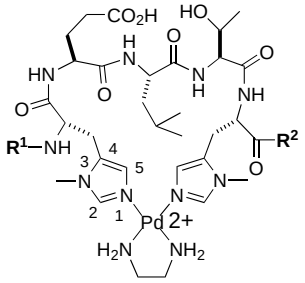
About 30% of all amino acids in proteins are found in α helices.[1] When buried in the hydrophobic interiors of proteins, helices are important in stabilizing tertiary structures, promoting protein folding, and molding active sites of enzymes. However, short peptides (< 15 amino acids) corresponding to such helices are usually unstructured outside of their hydrophobic protein environments.[2] Attempts to produce short, stable α helices by using noncovalent side chain constraints (e.g., salt bridges, hydrophobic interactions),[3a,b] covalent side chain linkers (e.g., disulfide,[3c] hydrazone,[3d] aliphatic,[3e] lactam[3f] bridges) have met with some success. However, little is known about the capacity of metal ions to stabilize α helicity in short peptides,[4] even though metals are often bound to the helical regions of metalloproteins.[5] Here we report that at 300 K $[Pd(en)]^{2+}$ induces helicity in 5-, 10-, and 15-residue nonhelical peptides corresponding to the Zn^{2+}-binding α helix of the active site of thermolysin (Figure 1). Formation of an unusual 22-membered macrocycle in the metallopentapeptides **1**–**3**, by metal



1 (**R**^{1} = Ac, **R**^{2} = NH_2)
2 (**R**^{1} = Ac, **R**^{2} = AVTDY-NH_2)
3 (**R**^{1} = Ac-IDVVA, **R**^{2} = AVTDY-NH_2)

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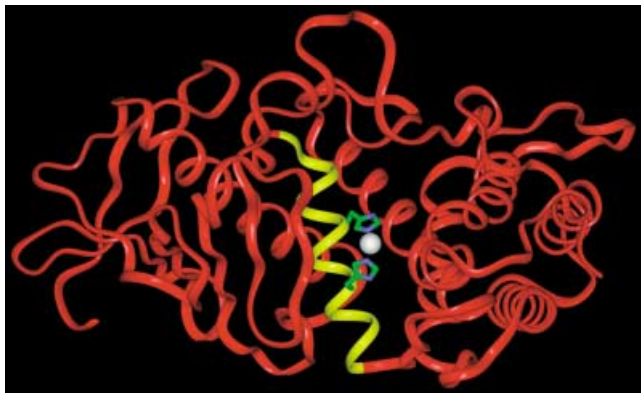

Figure 1. Crystal structure[6] of the metalloprotease thermolysin highlighting the α helix of the active site (yellow) containing two, $i,i+4$-spaced histidines (green/blue) bound through their N1 nitrogen atoms to tetrahedral Zn^{2+} (white).

coordination to two histidine residues spaced three residues apart (i.e. $i,i+4$), results in helical structures in solution that are analogous to those in the thermolysin crystal structure.[6]

Reaction of $[Pd(^{15}NH_2CH_2CH_2{}^{15}NH_2)(ONO_2)_2]$ with one equivalent of each of the following thermolysin fragments[7] Ac-H*ELTH*-NH_2 (**4**), Ac-H*ELTH*-AVTDY-NH_2 (**5**) and Ac-IDVVA-H*ELTH*-AVTDY-NH_2 (**6**) (Ac = acetyl) in DMF (or water) exclusively led to the respective metallopeptide products **1**–**3**, which were unambiguously characterized by multinuclear (^{1}H, ^{15}N, ^{13}C) and 2D NMR spectroscopy (see Supporting Information). Formation of a $[Pd(en)(peptide)]^{2+}$ product **1**, with 1:1 en:peptide stoichiometry, from **4** was supported by an unambiguously assigned single set of peptide ^{1}H NMR resonances (Figure 2a). The resonances of the imidazole protons H2 and H5 of both histidine residues (H*1H2, H*5H2, H*1H5, H*5H5) are shifted significantly after palladium addition. The ^{15}N NMR spectrum of **1** exhibited just two resonances (Figure 2b), both upfield of the resonance of $[Pd(^{15}NH_2CH_2CH_2{}^{15}NH_2)(solvent)_2]$ (~ −26 ppm; $\delta(NH_4^+) = 0.0$ ppm) and both with chemical shifts characteristic of inequivalent ethylenediamine nitrogen atoms *trans* to nitrogen donors.[8] The only other possible N donors (amide nitrogen atoms) were not coordinated to Pd since all amide-NH protons were accounted for in the one set of ^{1}H NMR resonances observed (Figure 2a).

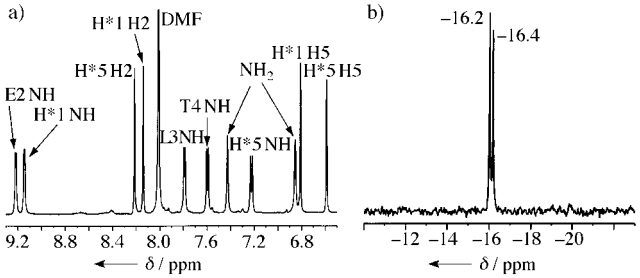

Figure 2. a) 500 MHz ^{1}H NMR spectrum (amide region) of reaction product **1** (obtained from ~3 μmol $[Pd(^{15}NH_2CH_2CH_2{}^{15}NH_2)(ONO_2)_2]$ and ~3 μmol **4** in 0.5 mL $[D_7]$DMF). b) 40.5 MHz ^{15}N NMR spectrum of the same mixture corroborating formation of **1**.

Alternative Pd coordination to the oxygen atoms of amide units or of Asp/Thr/Tyr side chains would instead be characterized by ^{15}N NMR chemical shifts of −24 to −26 ppm for the *trans*-en ligand.[8] ^{13}C NMR chemical shifts also indicated imidazole coordination to palladium, a large increase in δ_{C5} of each imidazole ring in **1** relative to **4** being diagnostic of N1 bound to Pd^{II}.[9] Finally, ROESY spectra and the structures calculated from the NMR data further confirmed the chemical identity of **1**.

Free peptide **4** was unstructured in solution as evidenced by 2D ROESY spectra which showed only intra-residue and sequential cross peaks (see Supporting Information). In contrast, metallopeptide **1** showed ^{1}H NMR parameters characteristic of defined structure, specifically of helicity (see Supporting Information). Helicity in **1** was supported by a) characteristic upfield shifts of the CHα signals[10] relative to free peptide **4**, b) multiple $^3J_{NHCH\alpha}$ coupling constants of ≤ 6 Hz,[11] c) amide-NH chemical shifts with low temperature dependence ($\Delta\delta/T \leq 4$ ppb K^{-1}), consistent with the presence of hydrogen bonds,[12] and d) characteristic[13] nonsequential cross peaks in the ROESY spectra ($d_{\alpha N(i,i+3)}$, $d_{\alpha N(i,i+4)}$, $d_{\alpha\beta(i,i+3)}$, Figure 3a,b). Variable temperature NMR data suggest the presence of three hydrogen-bonded amide protons (His5, Thr4, and Leu3), supportive of a 3_{10} helix, whereas only two hydrogen bonds (His5-NH···OC-His1, Thr4-NH···OC-Ac) would be expected for an α helix. On the other hand, the presence of $d_{\alpha N(i,i+4)}$ ROEs supports α rather than 3_{10} helicity for **1**.[13] Most likely there is rapid interconversion between the two helical forms,[14] but the metallacycle is certainly α-helical within longer peptides (see below).

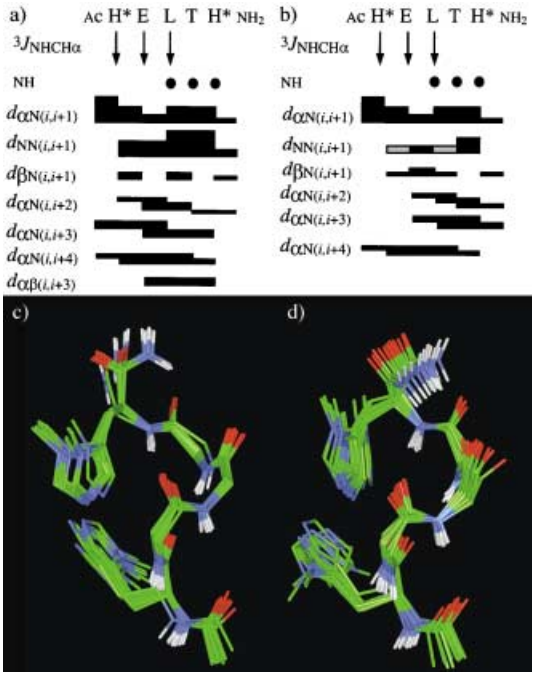

Figure 3. Top: Summary of sequential and medium-range ROE data for **1** in a) [D$_7$]DMF, b) H$_2$0/D$_2$O (9/1). Residues with $^3J_{NHCH\alpha} \leq 6$ Hz marked by ↓, amide protons with temperature coefficients $\Delta\delta/T = 4$ ppb K^{-1} by ●. The ROE intensities are symbolized by different bar thicknesses, which correspond to distances ≤2.7, ≤3.5, ≤5.0, and ≤6.0 Å. Shaded bars represent ROEs obscured by spectral overlap. Bottom: Backbone superimposition of the 14 lowest-energy refined structures calculated for **1** in c) [D$_7$]DMF and d) H$_2$0/D$_2$O (9/1). (average backbone pairwise rmsd 0.21 Å and 0.33 Å, respectively). Side chains of His* residues only are shown for clarity.

Structures were initially calculated for **1** in [D$_7$]DMF (74 ROE distance restraints: 35 intra-residue, 22 sequential, 17 medium-range; 3 backbone ϕ angles from $^3J_{NHCH\alpha}$ values) and H$_2$O/D$_2$O (9/1) (48 ROEs: 24 intra-residue, 15 sequential, 9 medium-range; 3 backbone ϕ angles) using a dynamic simulated-annealing and energy-minimization protocol in X-PLOR (3.851)[15] without explicit hydrogen bond or [Pd(en)$^{2+}$] restraints to prevent structure biasing. Since the N1 atoms of the two His* residues were correctly oriented and spaced to coordinate Pd in the initial calculations, they were constrained in the final calculations to within 2.7 Å of each other, this being the known separation between nitrogen atoms of N-Pd-N units (Pd-N 1.90 Å).[16] The resulting structures (Figure 3c,d) were helical and similar in the two solvents, although the structures were less ordered in water as indicated by the higher backbone root mean square deviation (rmsd). Their stability is attributed to His5-N1···Pd···N1-His1 bonds and intramolecular constraints imposed by hydrogen bonds.

Can the helical metallopentapeptide **1** propagate helicity when incorporated in longer peptides corresponding to the α helix in the active site of thermolysin? All of the NMR data for **1** suggest less helicity in water than in DMF. For this reason, and also because the corresponding thermolysin sequence is buried within the hydrophobic interior of the protein, we chose to examine helix induction by [Pd(en)]$^{2+}$ for the longer thermolysin fragments **5** and **6** in the aprotic solvent DMF, rather than in water.[17] We report that [Pd(en)]$^{2+}$ selectively coordinates N1 of the two $(i,i+4)$ histidine residues in **5** and **6** to exclusively form metallopeptides **2** and **3**, respectively, despite **5** and **6** being functionalized with many potential metal-binding sites. The chemical identities of metallopeptides **2** and **3** were confirmed using multinuclear and 2D NMR spectroscopy as described above for **1** (see Supporting Information).

2D ROESY spectra revealed that the free peptides **5** and **6** were unstructured in solution (see Supporting Information), whereas the metallopeptides **2** and **3** showed characteristic parameters for α-helical conformations. Both **2** and **3** exhibited many features that are consistent with helicity: upfield shifts of the CHα signals relative to the free peptides, low temperature dependence of the amide proton signals, low $^3J_{NHCH\alpha}$ coupling constants (≤6.0 Hz), medium-range NOEs (Figure 4a,b), and the calculated solution structures (Figure 4c–f). Multiple $d_{\alpha N(i,i+4)}$ NOEs, both within the metallacyclic components and throughout **2** and **3**, strongly favor α rather than 3_{10} helicity in these longer peptides.

The three-dimensional solution structures (in [D$_7$]DMF) for **2** (derived from 133 NOE distance restraints: 61 intra-residue, 46 sequential, 26 medium-range, and 5 torsion angle restraints: for $^3J_{NHCH\alpha} \leq 6$ Hz, $\phi = -60$ to $-25°$) and **3** (derived from 234 NOE distance restraints: 95 intra-residue, 65 sequential, 74 medium-range and 10 ϕ angle restraints) were calculated as for **1** using X-PLOR.[15] The backbone superimposition of the final 14 lowest-energy structures for **2**

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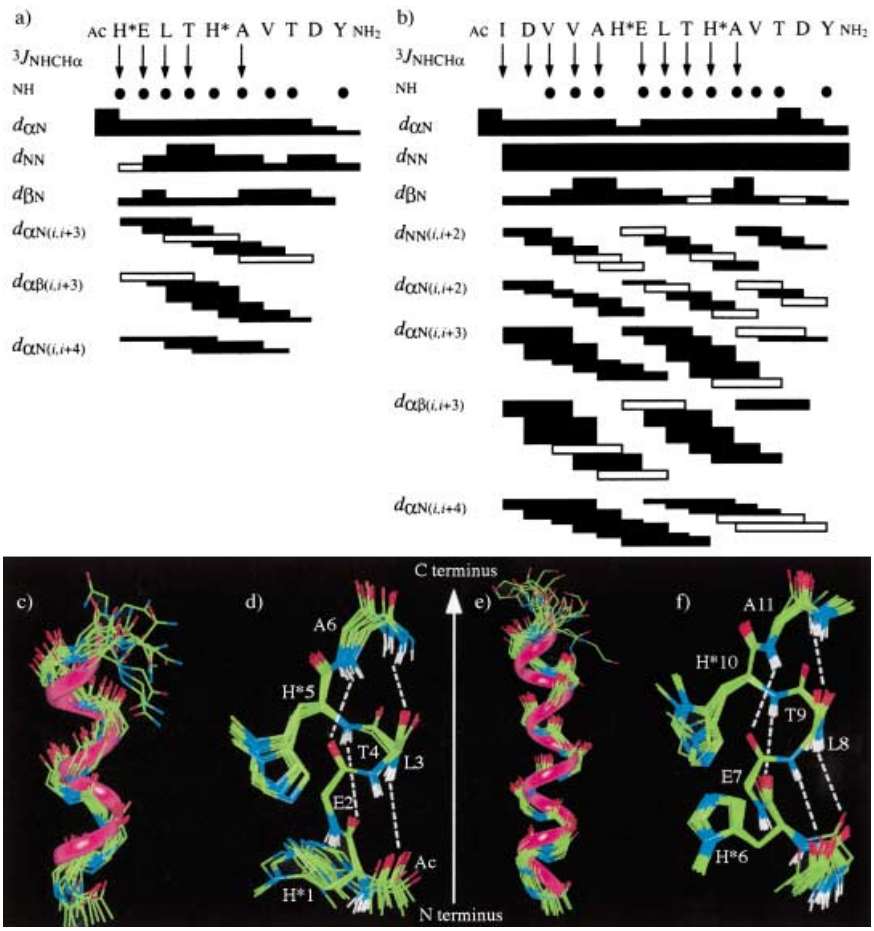

Figure 4. Top: Summary of sequential and medium-range NOE data for a) **2** and b) **3** in [D_7]DMF. (cf. Figure 3). Bottom: Backbone superimposition of c) the 14 lowest-energy refined structures of **2** (green; average backbone pairwise rmsd 1.37 Å; side chains omitted for clarity), d) residues H1* to A6 showing the helical metallacycle, four intrahelical hydrogen bonds, and imidazole locations, e) the 20 lowest-energy refined structures of **3** (green; average backbone pairwise rmsd 0.81 Å), f) residues H1* to A11 showing the helical metallacycle, five intrahelical hydrogen bonds, and imidazole locations. The purple ribbon in (c) and (e) is the superimposed backbone of the analogous α helix of thermolysin.[6]

(Figure 4c,d) indicates the formation of an α helix that closely mimics the α-helical counterpart in thermolysin, except for some fraying at the exposed C terminus. Its cyclic metallopeptide component forms a well defined α-helical turn featuring the hydrogen bonds Ac-CO···HN-Thr4, His1-CO···HN-His5, Glu2-CO···HN-Ala6, Leu3-CO···HN-Val7, and Thr4-CO···HN-Thr8, the first four being shown in Figure 4d.

The backbone superimposition of the final 20 lowest-energy structures representing **3** (Figure 4e,f) also indicates a regular α helix which closely mimics its 15-residue α-helical counterpart in thermolysin, except for some fraying at the C terminus. Some of the hydrogen bonds in the metallacycle are marked in Figure 4f. The metallacycle is the most highly structured α-helical region of **3** and does propagate α helicity in both directions towards the N and C termini. In contrast to the disorder at the C terminus, there was ordered α-helical structure extending to the N terminus of **3**. This difference may be the result of better C-terminal α helix nucleation by the metallacycle, or alternatively the N-terminal sequence may simply have a lower helical propensity.

It has been demonstrated here that Pd^{2+} preferentially binds imidazole N1 atoms over alternative peptide components including carboxylate, alcohol, amide, and phenol units. The resulting 22-membered metallacycle, while uncommon for inorganic complexes in which metals (even Pd^{2+}) normally favour 5- or 6-membered rings,[18] is not an uncommon chelate ring size in metalloproteins.[19] Although structure calculations on small cyclic peptides can be notoriously misleading, because they assume the presence of a single conformation when many rapidly interconverting structures are more likely in solution, we have clearly identified helicity for **1** in both DMF and water. While the distinction between α and 3_{10} helicity was not unequivocal for **1**, the fact that we do observe α-helix induction in the longer peptides **2** and **3** (incorporating **1**) supports α helicity in **1** as well.

Helicity in the $[Pd(en)(HxxxH)]^{2+}$ metallacycle enables it to act as a template, propagating α helicity through at least one additional helical turn (3.6 residues) at either side of the metallacycle. Disorder at the C terminus of peptides **2** and **3** may have obscured the true extent of this helix induction, which might be transmitted even further in longer peptides. No compound has been reported to nucleate an α helix from the C terminus of a short peptide, and only a few N-terminal nucleators have been described,[20] those being organic scaffolds that are difficult to synthesize and require covalent linking to peptides thereby limiting their applications. Metallacycles like $[Pd(en)(HxxxH)]^{2+}$ may find wider applications due to their ease of formation and successful helix propagation in both N- and C-terminal directions. It remains to be seen whether moieties such as $[Pd(en)]^{2+}$ can be used as metal clips to reversibly fold peptide and protein domains. Although we have not observed hydrolysis of these histidine-containing peptides in solution, this side reaction has been reported in other systems[21] and could potentially affect the utility of these metal clips.

HxxxH is a common signature in proteins and raises the possibility that metal ions may play important roles as helix-folding templates. This could be true not only in metalloproteins, but also in other proteins to which first-row transition metals may only be transiently coordinated. The use of the diamagnetic $[Pd(en)]^{2+}$ moiety has enabled us to detect thermodynamically stable structures in solution by

NMR spectroscopy. More labile metal complexes only form transient metallopeptides that are kinetically labile and more difficult to study. For example, adding Zn^{2+} to solutions of these peptides results only in broad NMR signals, and CD spectra showed negligible structure. Less labile metal complexes may alternatively capture donor atoms other than N1 of a His imidazole ring, forming kinetically inert nonhelical metallopeptides. This work demonstrates that small metallopeptides may have potential for seeding α helicity in peptides and proteins, for studying α-helical turns outside proteins, and for producing artificial models of the active sites of metalloproteins.

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